Mapping the binding site for the GTP-binding protein Rac-1 on its inhibitor RhoGDI-1

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Background: Members of the Rho family of small GTP-binding proteins, such as Rho, Rac and Cdc42, have a role in a wide range of cell responses. These proteins function as molecular switches by virtue of a conformational change between the GTP-bound (active) and GDP-bound (inactive) forms. In addition, most members of the Rho and Rac subfamilies cycle between the cytosol and membrane. The cytosolic guanine nucleotide dissociation inhibitors, RhoGDIs, regulate both the GDP/GTP exchange cycle and the membrane association/dissociation cycle.

Results: We have used NMR spectroscopy and site-directed mutagenesis to identify the regions of human RhoGDI-1 that are involved in binding Rac-1. The results emphasise the importance of the flexible regions of both proteins in the interaction. At least one specific region (residues 46–57) of the flexible N-terminal domain of RhoGDI, which has a tendency to form an amphipathic helix in the free protein, makes a major contribution to the binding energy of the complex. In addition, the primary site of Rac-1 binding on the folded domain of RhoGDI involves the β4–β5 and β6–β7 loops, with a slight movement of the 3_{10} helix accompanying the interaction. This binding site is on the same face of the protein as the binding site for the isoprenyl group of post-translationally

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Conclusions: Isoprenylated Rac-1 appears to interact with three distinct sites on RhoGDI. The isoprenyl group attached to the C terminus of Rac-1 binds in a pocket in the folded domain of RhoGDI. This is distinct from the major site on this domain occupied by Rac-1 itself, which involves two loops at the opposite end to the isoprenyl-binding site. It is probable that the flexible C-terminal region of Rac-1 extends from the site at which Rac-1 contacts the folded domain of RhoGDI to allow the isoprenyl group to bind in the pocket at the other end of the RhoGDI molecule. Finally, the flexible N terminus of RhoGDI-1, and particularly residues 48–58, makes a specific interaction with Rac-1 which contributes substantially to the binding affinity.

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Introduction

The Rho family of small GTP-binding proteins includes the isoforms of Rho, Rac, Cdc42 and TC10 [1,2] which have roles in a variety of processes: regulation of the actin cytoskeleton in response to external stimuli; activation of MAP kinase cascades; cell transformation by Ras; cell-cycle progression; and the activation of NADPH oxidase [2–9]. The cycling of these proteins between the GTP-bound and GDP-bound forms is controlled by guanine nucleotide exchange factors (GEFs), which catalyse the exchange of GTP for GDP, and the GTPase-activating protein (GAPs), which accelerate GTP hydrolysis [1,2]. In addition, their activity is regulated by the RhoGDIs [10,11], which inhibit release of nucleotides from the GTPase [11,12] and also control partitioning of the GTPase between the cytosol and membrane [13–15]. Three human RhoGDIs have been identified. The ubiquitously expressed RhoGDI-1 [11], the form studied here, and D4/Lys-GDI (RhoGDI-2) [16,17], expressed in haematopoetic cells, both have a broad range of activity towards the Rho proteins. In contrast, RhoGDI-3 [18] is specific for RhoB and RhoG, and unlike the other two cytoplasmic forms is associated with the membrane or possibly the cytoskeleton. Although the binding of a Rho family GTPase to RhoGDI appears to lock it into its inactive state, in some cases (e.g. in the regulation of ezrin/radixin/moesin [19] and phospholipase Cβ2 [20]) the GTPase has been reported to bind to an effector molecule

as a complex with RhoGDI, suggesting that RhoGDI may also have a role in directing the GTPase to the effector.

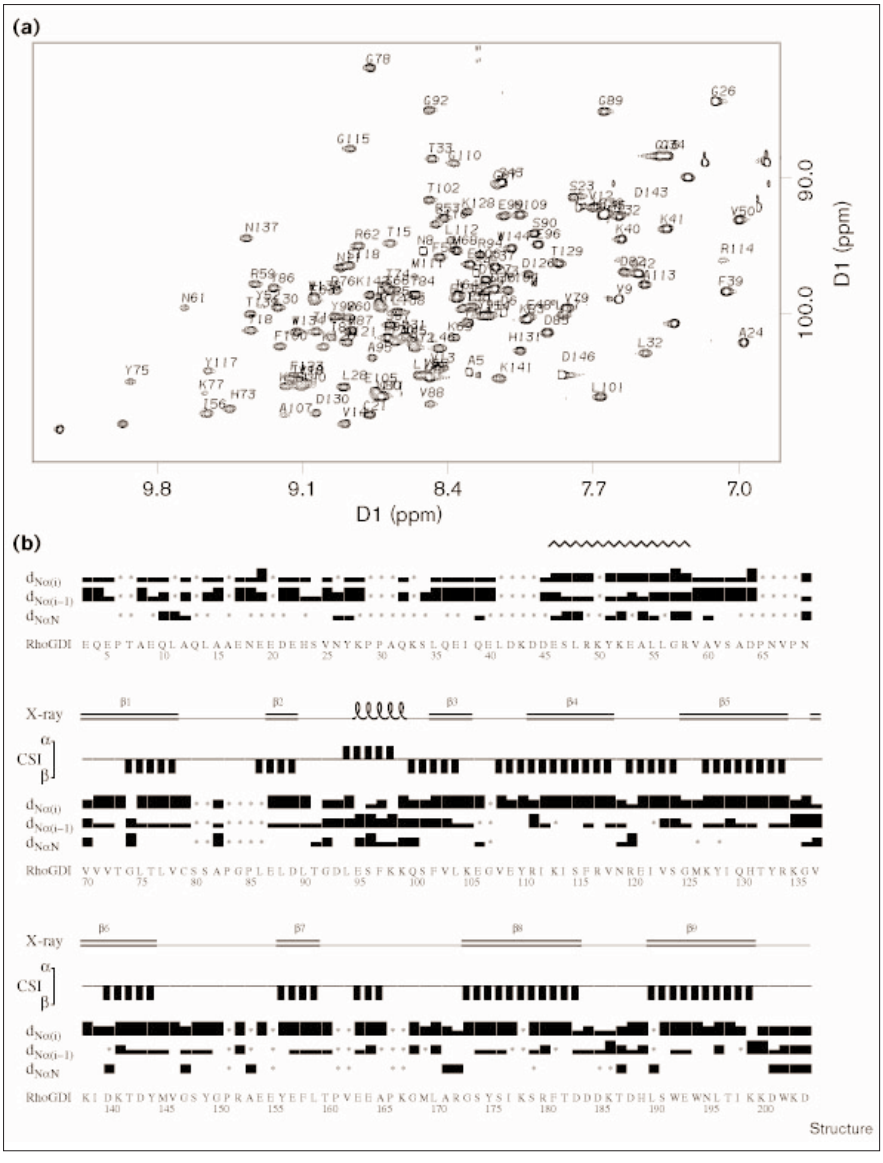
We have recently determined the structure of human RhoGDI-1 by a combination of nuclear magnetic resonance (NMR) spectroscopy and crystallography [21]. The ^{15}N–^{1}H HSQC spectrum of full-length RhoGDI-1 showed two types of cross-peaks: between 50 and 60 of the backbone amide cross-peaks had much narrower linewidths than the others. These sharp cross-peaks were removed by selective cleavage with trypsin at Arg58. This indicated that RhoGDI consists of two domains — a flexible N-terminal domain of about 60 amino acids and a folded domain. Similar observations have been made with a fragment of bovine RhoGDI comprising residues 23 to 204 — RhoGDI(23–204) [22]. The crystal structure [21] of human RhoGDI(59–204) and the solution structure [22] of bovine RhoGDI(60–204) showed that the truncated form has an immunoglobulin fold. We now report the use of the assigned ^{1}H, ^{13}C and ^{15}N resonances of human RhoGDI-1 to identify the residues that are involved in its interaction with Rac-1.

Results and discussion

The flexible N-terminal domain of RhoGDI

Figure 1a shows the ^{1}H–^{15}N correlation spectrum of human RhoGDI(59–204), uniformly labelled with ^{15}N. The backbone resonances were assigned using two separate samples, one of the truncated (59–204) protein and the other of the full-length protein. The tendency of the

Figure 1



NMR data of RhoGDI-1. **(a)** A ^{1}H–^{15}N correlation spectrum of uniformly [^{13}C,^{15}N]-labelled RhoGDI-1, with resonance assignments indicated. **(b)** Secondary structure of RhoGDI-1 as identified from the NMR data. From bottom to top: the amino acid sequence of RhoGDI-1; sequential and intraresidue NOEs (the heights of the solid bars are proportional to the intensities of the NOE cross-peaks; asterisks indicate NOEs that have not been identified due to resonance overlap and/or missing assignments); the consensus chemical-shift index; and the secondary structure in the X-ray structure (double lines indicate β strands and helical symbols indicate the 3_{10} helix). The sequence in the N-terminal domain identified by NMR as having a tendency to form a helix is indicated by a zig-zag line.

full-length protein to cleave meant that this latter sample was used to obtain the assignments of the N-terminal domain only; the high intensities of the resonances from the flexible domain allowed data acquisition for this domain to be completed more quickly than for the folded domain. The resonances were assigned on ^{13}C,^{15}N-labelled samples using a suite of triple-resonance experiments as described in the Materials and methods section; we showed earlier that the signals of the folded domain were virtually unaffected by removal of the flexible N-terminal domain [21]. The information on the secondary structure in the N-terminal domain was derived from the intra-residue and sequential nuclear Overhauser effects (NOEs) involving backbone amide protons and from the carbon chemical-shift index [23], as shown schematically in Figure 1b. For the folded domain, the secondary structure deduced from the NMR data is in good agreement with the nine β strands and a short 3_{10} helix identified in our X-ray crystal structure of human RhoGDI(59–204) [21] and with the solution structure of bovine RhoGDI(60–204) [22], which differs from the human protein in only six amino acid substitutions; there are only minor differences (plus or minus two residues) between the structures at the start and end points of some of the β strands.

Most of the N-terminal domain has a flexible random-coil structure, as indicated by the observed chemical shifts and NOEs as well as the narrow linewidths. However, a short stretch of this domain, residues 48–58, shows a pattern of sequential and intraresidue NOEs characteristic of a helical structure (Figure 1b); no medium-range NOEs characteristic of a helical fold were observed, and we conclude that this part of the sequence has a tendency to form a helix, but exists in solution in equilibrium between helical and random-coil conformations.

The binding site for the GTPase isoprenyl group

The enzyme geranylgeranyl transferase I recognises the C-terminal Cys-X-X-Leu sequence of the Rho family GTPases and transfers a geranylgeranyl group to the cysteine residue. This isoprenylation of the GTPase is important for tight binding to RhoGDI [24]. Examination of the structure of the folded domain of RhoGDI revealed the existence of a largely hydrophobic cavity between the two β sheets of the immunoglobulin fold [21,22], which was proposed to be the binding site for this isoprenyl group. Clear evidence for this was reported [22], which showed that the chemical shifts of the resonances of a number of residues in and near this cavity in bovine RhoGDI(60–204) were affected by addition of a farnesylated peptide. We have carried out similar experiments with human RhoGDI, using *N*-acetyl farnesyl cysteine. The addition of 0.2–1 M equivalents (100–500 μM) of *N*-acetyl farnesyl cysteine to either the truncated or intact human RhoGDI produces a limited number of clear changes (greater than the linewidth of the cross-peak) in the ^{15}N and/or ^{1}H chemical shifts of specific residues. Progressive changes in chemical shift were observed on titration of the ligand into the protein sample (data not shown), implying that the free and complexed proteins are in fast exchange on the NMR timescale, and allowing the assignment of the protein resonances in the complex to be obtained from the assignments of the free protein. Addition of an excess of *N*-acetyl *S*-farnesyl cysteine or of even small amounts of *N*-acetyl *S*-geranylgeranyl cysteine resulted in general nonspecific line-broadening of the RhoGDI signals, presumably due to a detergent-like action. However, the specific changes in chemical shift seen at low concentrations of *N*-acetyl *S*-farnesyl cysteine reflect direct protein–ligand contacts and/or small changes in conformation on complex formation.

In the full-length RhoGDI(1–204), the chemical-shift changes on addition of *N*-acetyl farnesyl cysteine occur only in the resonances of residues arising from the folded part of the molecule; none of the intense signals from the flexible N-terminal region is affected. The changes seen with the full-length protein are identical to those seen on adding *N*-acetyl farnesyl cysteine to the truncated form, RhoGDI(59–204), and include the backbone amide resonances of 19 residues: Ser81, Leu104, Lys105, Val108, Arg111, Gln130, His131, Tyr133, Glu163, Glu164, Ala165, Lys167, Gly168, Met169, Ala171, Gly173, Ser174, Tyr175 and Lys178 (Figure 2). The list of affected residues is closely similar (but not wholly identical) to that reported [22] for the binding of a farnesylated peptide to the bovine protein. These residues are clustered around the cavity between the β sheets (Figure 3), and include six residues previously identified [21] as lining the cavity. Of the 19 residues affected in human RhoGDI-1, 12 are surface residues and seven are largely or completely buried. The fact that some buried residues are affected indicates that the chemical-shift changes cannot arise exclusively from direct effects of ligand binding, but must also reflect some degree of structural change. The surface residues affected comprise largely residues in the loops between β strands 3 and 4 (residues 105–111) and between β strands 7 and 8 (residues 160–172); some movement of these loops on ligand binding could account for the effects on buried residues and on four residues on the opposite face of the protein, Gly173 and Ser174 in β strand 8 and His131 and Tyr133 in the neighbouring β strand 5. The residues affected are predominantly uncharged; Figure 3a shows that the patch of affected residues includes a nonpolar surface patch at the C-terminal end of the molecule that is connected to the cavity.

The NMR evidence thus clearly defines the location of the binding site for *N*-acetyl farnesyl cysteine, and suggests that the cavity in RhoGDI and the neighbouring hydrophobic surface patch form the site of interaction of the C-terminal isoprenyl group of Rac, an interaction

Figure 2

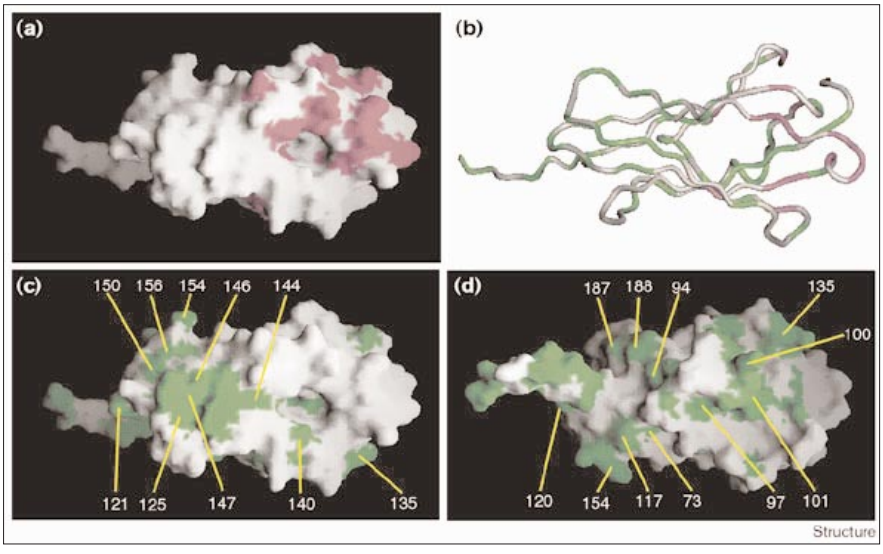
Amino acid sequence alignment for members of the RhoGDI family. Residues identical in at least five of the eight sequences are shown in bold. Residues with amide resonances affected by ligand binding are indicated in the sequence of human RhoGDI-1: residues affected by *N*-acetyl farnesyl cysteine binding are shown in blue, residues affected by Rac-1 binding are in red, and residues affected by both ligands are in green. Proline residues, which do not have amide resonances, and four residues for which amide resonances have not been assigned are shown in italics. The secondary structure elements in the folded domain are indicated by β for β strands and 3 for the 3_{10} helix. The sequence alignment was carried out using CLUSTAL W, with a minor manual adjustment to avoid an insertion in the 3_{10} helix. The sequences shown are: hRGDI-1, human RhoGDI-1; bRGDI, bovine RhoGDI; hRGDI-2, human RhoGDI-2 (equivalent to D4/Ly GDI); mRGDI-2, mouse RhoGDI-2; hRGDI-3, human RhoGDI-3 (equivalent to GDIγ); mRGDI-3, mouse RhoGDI-3; cRGDI, *C. elegans* RhoGDI homologue; yRGDI, *S. cerevisiae* RhoGDI.

which makes an important contribution to the tight binding of the two proteins to one another.

Residues affected by the binding of Rac-1

We showed earlier that the addition of recombinant non-isoprenylated full-length Rac-1 to ^{15}N-labelled full-length RhoGDI-1 leads to specific changes in the chemical shift of a limited number of cross-peaks in the ^{15}N–^{1}H heteronuclear single quantum coherence (HSQC) spectrum [21] showing that non-isoprenylated Rac-1 binds specifically to RhoGDI, although much more weakly than the geranylgeranylated protein. When Rac-1 was added to truncated RhoGDI(59–204), the spectrum of the latter was unaffected [21], demonstrating the importance of the flexible N-terminal region of RhoGDI in binding Rac-1. An identification of the binding site for Cdc42 on bovine RhoGDI, at the opposite end of the structured domain to the isoprenyl-binding pocket, has previously been proposed [22]. This was based on the effects of substoichiometric amounts of Cdc42 on the intensities of cross-peaks, assumed to reflect linewidth changes due to exchange broadening, in the ^{1}H–^{15}N HSQC spectrum of RhoGDI(23–204). This approach to mapping the interaction surface between the two proteins

Figure 3



Molecular surface of the folded domain of RhoGDI coloured to indicate residues with backbone amide ^{1}H and/or ^{15}N resonances that show significant changes in chemical shift on ligand binding. **(a)** Residues affected by binding *N*-acetyl *S*-farnesyl cysteine (magenta). **(b)** Ribbon diagram of the backbone of RhoGDI in the same orientation as (a). **(c,d)** Residues affected by binding Rac-1 (green). The view in (d) differs from those in (a–c) by a 180° rotation about the horizontal axis. (Parts (a), (c) and (d) were created using the program GRASP [33].)

has some disadvantages: it requires demonstration that exchange between the complexed and free protein is reasonably fast, and the origin of the line-broadening (leading to loss of intensity) may be either or both a decrease in mobility (in this case, notably in the N-terminal domain) or exchange broadening associated with a change in chemical shift.

We have now established conditions under which a reasonably stable stoichiometric complex between RhoGDI and Rac-1 can be formed; exchange between the free and complexed states is slow, permitting the direct observation of the resonances of the complex. Using the tranverse relaxation-optimised spectroscopy (TROSY) technique [25], a ^{1}H–^{15}N correlation spectrum was obtained which contained sharp resonances from this 46 kDa complex (Figure 4). The stability of the complex is limited by a very slow cleavage (over several days, presumably by very small amounts of contaminating proteases) of full-length RhoGDI in its N-terminal region; this is somewhat faster in complexed than in free RhoGDI. This cleavage leads to a dissociation of the complex, and the ^{15}N–^{1}H HSQC spectrum of cleaved RhoGDI liberated in this way is identical to that of the free truncated protein. This confirms the importance of the N-terminal domain for binding to Rac-1.

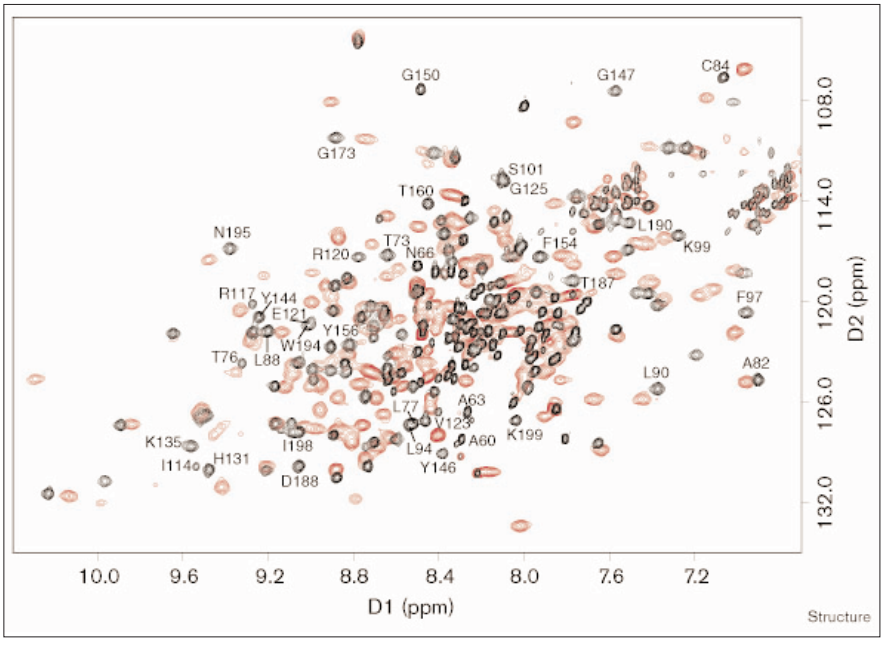
In line with these observations, and in contrast to the effects of *N*-acetyl farnesyl cysteine, the effects of Rac-1 binding involve resonances from both the flexible N-terminal domain and the structured domain. The resonances of RhoGDI in the complex are not yet assigned, so that the magnitude of the shifts of individual resonances cannot be accurately determined. We have used the 'minimum chemical shift' approach [26,27], in which the chemical-shift difference from a given cross-peak in the free protein to the closest cross-peak in the complex is calculated, as described in the Materials and methods section. This approach can provide a reliable identification of the interaction site(s) [27,28], although caution is clearly required in using it in very crowded regions of the spectrum; work is currently in progress to assign the resonances of both RhoGDI-1 and Rac-1 in the complex.

Contacts in the folded domain

Of 134 residues in the folded domain, 49 residues (70–204) of full-length RhoGDI-1 (36%) show changes in backbone amide chemical shifts on Rac-1 binding which are significant by the criterion described in the the Materials and methods section. These residues are distributed quite widely through the sequence (Figure 2) and on the surface of the protein (Figure 3), but on detailed examination a pattern does emerge. On the same surface of the molecule as the isoprenyl-binding pocket (Figure 3a), two groups of residues affected by Rac-1 binding can be discerned: a small number of residues near the isoprenyl-binding pocket and a much larger patch at the other end of the domain. Of the 19 residues affected by N-acetyl farnesyl cysteine binding only four are also affected by Rac-1 binding (Figure 2). This suggests that the non-isoprenylated but full-length Rac-1 protein makes only minimal contact with the part of RhoGDI which binds the isoprenyl group. Further evidence for this was obtained from

Figure 4

Binding of RhoGDI-1 to Rac-1. ^{1}H–^{15}N TROSY spectra of RhoGDI-1 alone (0.25 mM; black) and in the presence of Rac-1 (0.3 mM; red).



experiments in which the water-soluble nitroxide spin-label TEMPO was added to a sample of the complex. Marked line-broadening, indicative of accessibility to the spin-label, was observed for residues surrounding the isoprenyl-binding cavity (data not shown).

The large distinct surface patch of residues affected by Rac-1 binding at the other end of the folded domain from the isoprenyl-binding pocket (Figure 3c) consists primarily of residues in the loops between β strands 6 and 7 (residues 143–156) and between β strands 4 and 5 (residues 119–125); the latter stretch of residue forms an extension across to the point where the flexible N terminus enters the folded domain. It is notable that the residues in these two surface loops are substantially conserved in the sequences of all mammalian RhoGDIs and to a lesser extent in those of the *Caenorhabditis elegans* and *Saccharomyces cerevisiae* proteins (Figure 2); in the β6–β7 loop in particular, all but three of the 13 residues are affected by Rac binding and all but two are conserved in at least five of the RhoGDI sequences in Figure 2. We therefore conclude that these two loops form a primary site of interaction of Rac-1 with the folded domain of RhoGDI-1.

Gosser *et al.*, [22] also identified these loops as being affected by Cdc42 binding to bovine RhoGDI, but reported a much larger patch of affected residues, including residues in the β8–β9 loop (181–189). Although we see changes in β strand 9, primarily in buried residues (see below), only two residues in the preceding loop (Thr187 and Asp188) are affected in our experiments, and these are on the opposite face of the molecule. It remains to be established whether this difference represents a real difference in the mode of binding of Cdc42 and Rac-1 to RhoGDI, or only reflects the methods used. For example, line-broadening of these residues could arise from a decrease in mobility of this loop rather than from large chemical-shift changes.

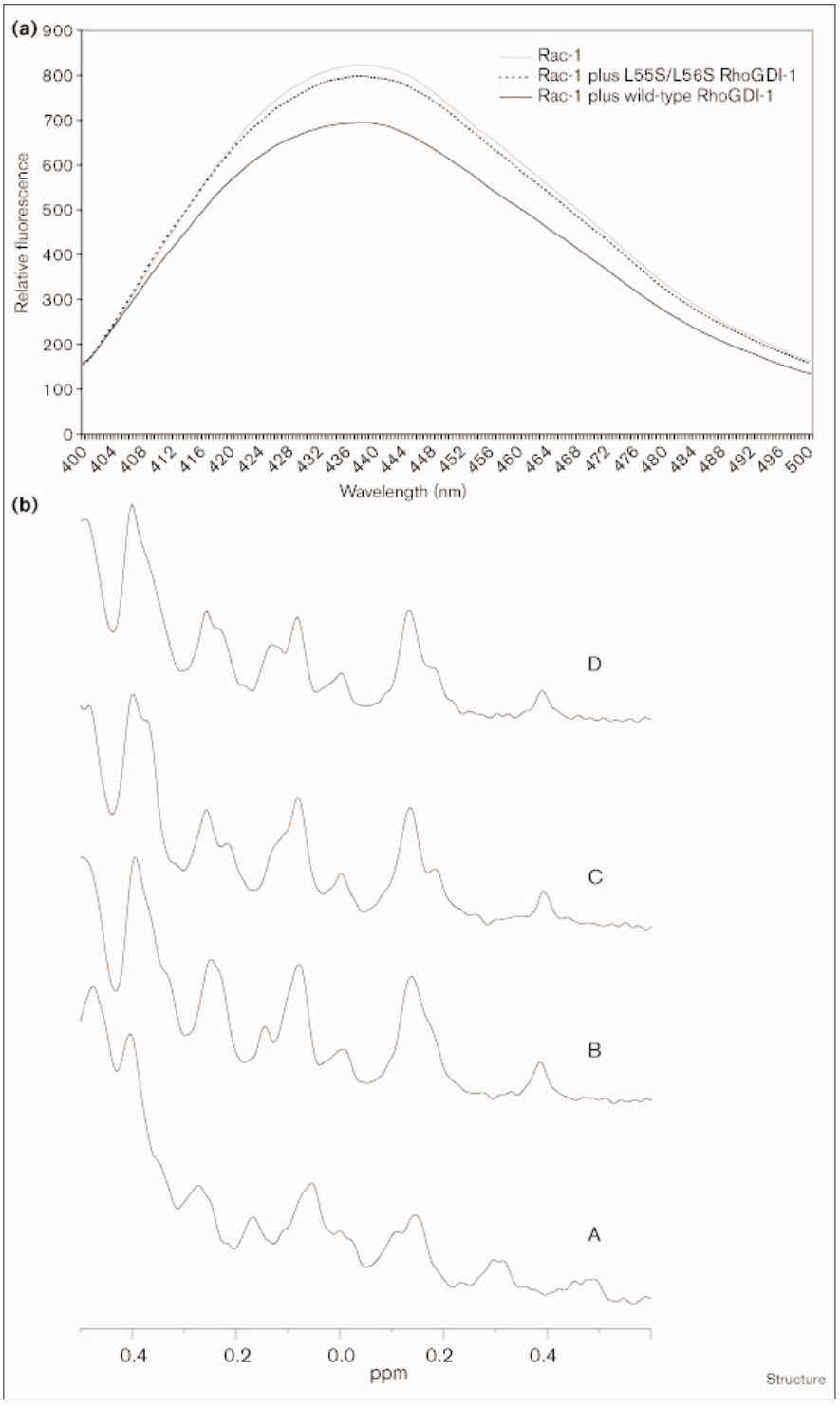
Apart from the residues in this major patch (Figure 3c), a number of buried residues and residues on the opposite face of the protein (Figure 3d) are also affected by Rac-1 binding. The affected surface residues shown in Figure 3d fall into two groups. Firstly, some residues in the β4–β5 and β6–β7 loops discussed above, together with two residues in the β8–β9 loop, surround the point at which the flexible N-terminal domain joins the folded domain. Secondly, a number of surface residues in contact with the short 3_{10} helix are affected. It is notable that several residues in β strand 9 (residues 193–199) are also affected by Rac-1 binding; these residues are buried in the RhoGDI structure and lie beneath the 3_{10} helix. In order to establish whether the effects of Rac-1 binding on this part of the molecule are direct or indirect, we constructed a mutant of RhoGDI-1 in which Asp93 and Glu95 were replaced by asparagine and glutamine, respectively. These two residues are located near the N-terminal end of the 3_{10} helix, in a region where several surface residues are affected by Rac-1 binding. However, the double mutant RhoGDI D93N/E95Q showed an affinity for N-methylanthraniloyl GDP (mantGDP)–Rac-1 indistinguishable from that of the wild-type protein. These observations suggest that the primary Rac-1 binding site on the folded domain of RhoGDI involves the β4–β5 and β6–β7 loops, and that some degree of structural change, perhaps involving a slight movement of the 3_{10} helix, accompanies the interaction.

Interaction of Rac-1 with the flexible N-terminal domain

On addition of Rac-1 to RhoGDI-1, almost half the sharp amide resonances arising from the N-terminal domain show significant changes in chemical shift (Figure 4). With very few exceptions, however, (see below) they remain markedly sharper than resonances from the folded domain, demonstrating that most of the N-terminal ~70 residues retain substantial mobility in the complex. The first 22–25 residues of RhoGDI are not required for binding Cdc42 [22,29], although similar data for Rac binding have not previously been reported and there is little sequence conservation among these residues; nonetheless, several residues show significant changes in chemical shift. This suggests the possibility of a weak interaction between these residues and Rac-1, most probably with a rapid equilibrium between conformations in which some of the first 25 residues of RhoGDI make contact with Rac-1 and conformations in which they do not.

There are clear-cut effects of Rac-1 binding on two regions of the N-terminal domain of RhoGDI (Figure 2), residues 22–41 and 45–60. In the first of these regions, 17 residues changes in amide chemical shift (discounting two proline residues). This part of the sequence shows a moderate degree of conservation, 47% of the residues being conserved in at least five of the sequences in Figure 2. In the second region, residues 45–60, 14 residues show changes in chemical shift; there is greater conservation in this region with 73% of the residues being identical in at least five RhoGDI sequences. In addition, marked line-broadening effects are seen in the spectrum of the selectively ^{15}N-leucine-labelled protein for the amide resonances of Leu48, Leu55 and Leu56, but not, for example, for Leu41. Similar broadening is seen for the resonance of Gly57, which is well resolved in the spectrum of the uniformly labelled protein. This combination of shifts and line-broadening clearly suggests that this part of the N-terminal domain interacts strongly with Rac-1. It is notable that this region includes residues 48–58, which show a tendency to form a helix in free RhoGDI-1. In contrast, no NOEs suggestive of any nascent secondary structure were observed for residues 22–41. Modelling the putative helix for residues 48–58 shows that it would have

Figure 5



Comparison of wild-type and mutant RhoGDI-1 binding to Rac-1. **(a)** Fluorescence emission spectrum of the mantGDP–Rac-1 complex (1 μM) alone, and in the presence of wild-type RhoGDI-1 (12 μM) or L55S/L56S RhoGDI-1 (12 μM). **(b)** Effects of RhoGDI-1 binding on the high-field methyl region of the ^{1}H NMR spectra. The spectrum of a mixture of wild-type RhoGDI-1 and Rac-1 (A) is clearly different from the sum of the spectra of the two individual proteins (B), showing that a complex is formed. In contrast, for L55S/L56S RhoGDI, the spectrum of the mixture (C) is identical to the sum of the individual spectra (D), showing that there is no significant interaction.

an amphipathic character with a nonpolar face containing the conserved leucine residues that show clear line-broadening, suggesting that this face of the helix might interact with Rac-1.

To investigate the importance of this possible interaction, we constructed mutants in which Leu55 and Leu56 of RhoGDI-1 were both replaced by the polar residues serine and asparagine. Both L55S/L56S and L55N/L56N

double mutants showed dramatically lower affinities than wild-type RhoGDI-1 for Rac-1 (Figure 5). Wild-type RhoGDI-1 bound to non-isoprenylated Rac-1 with a K_d of 1.66 (± 0.07) μM, but neither of the RhoGDI-1 mutants quenched the fluorescence of mant-GDP–Rac-1 (Figure 5a). Furthermore, the NMR spectrum of a mixture of Rac-1 with mutant RhoGDI-1 was simply the sum of the spectrum of the two components (Figure 5b), showing that they do not interact even at millimolar concentrations. The substitution of Leu55 and Leu56 in the N-terminal domain with polar residues thus decreases the affinity of RhoGDI-1 for Rac-1 by at least four orders of magnitude. These results demonstrate that residues 46–58 form a primary site of interaction between RhoGDI-1 and Rac-1. Residues 27–41 are clearly also affected by the interaction, but the exact nature of their involvement in complex formation is yet to be determined.

Biological implications

Members of the Rho family of small GTPases are involved in many cellular processes. In addition, the GTPase Rac has a specific role in the activation of the phagocytic NADPH oxidase. These small GTPases are regulated by the cytosolic guanine nucleotide dissociation inhibitors, RhoGDIs, in two ways: by inhibiting the dissociation of nucleotides from the GTPase, and by regulating its association with and dissociation from the membrane.

Our studies show that isoprenylated Rac-1 can interact with three distinct sites on RhoGDI-1, implying that (at least) three distinct sites on Rac-1 must be involved in the interaction. The isoprenyl group on Rac-1 binds in a hydrophobic pocket in the folded domain of RhoGDI-1, which is distinct from the major site on this domain occupied by Rac-1 itself. The N-terminal flexible region of RhoGDI-1, in particular residues 48–58, makes a major contribution to the binding energy of the complex, possibly interacting with residues in the mobile switch regions of Rac-1. The multiple binding regions fit a model where Rac-1 and RhoGDI-1 can interact with one another even when Rac-1 is bound to the membrane. The initial binding of the N-terminal region of RhoGDI-1 would be followed by a structural rearrangement resulting in the release of Rac-1 from the membrane, and the insertion of the isoprenyl group of Rac-1 into the hydrophobic pocket of RhoGDI-1. This mode of binding enables RhoGDI-1 to play an important role in the regulation of both the GDP/GTP exchange and the membrane association/dissociation cycles.

The importance of the N-terminal region for tight binding to Rac-1 demonstrated here parallels observations with RhoGDI-2. In RhoGDI-2, cleavage by interleukin-1β-converting enzyme effectively removes the equivalent N-terminal region and the resulting protein is unable to bind to Rho GTPases [30]. The present results also emphasise the importance of the flexible regions of the two proteins in the formation of a tightly bound Rac–RhoGDI complex.

Materials and methods

The cDNA coding for residues 59–204 of RhoGDI-1 was transferred into the *Nde* I and *Bam* HI sites in the expression vector pET11a (Pharmacia) and the construct transformed into *Escherichia coli* BL21(DE3). The transformant was grown on a defined medium containing 4 g/L glucose and 1 g/L ammonium chloride. [^{15}N]-labelled and [^{13}C,^{15}N]-labelled protein were prepared by using 99% [^{15}N]-NH_4Cl and 99% [^{13}C]-glucose (both from Isotec Inc.) as appropriate; for [^{2}H,^{15}N]-labelled protein, cells were grown in minimal media in $^{2}H_2O$. The protein was purified using a Pharmacia SP Sepharose cation exchange column, eluting with a linear gradient of 0–1 M NaCl in buffer A (50 mM sodium phosphate, 1 mM EDTA, 1 mM dithiothreitol [DTT] pH 5.8). The protein was further purified by size-exclusion chromatography using a Pharmacia Superdex 75 column in buffer A. Fractions containing pure truncated RhoGDI-1 were pooled and concentrated using an Amicon stirred cell. The protein was found to be homogeneous, as judged by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE), N-terminal sequencing and mass spectrometric analysis; the latter techniques confirmed that the protein corresponded to RhoGDI(59–204), with two extra methionine residues at the N terminus.

Full-length RhoGDI was expressed in the expression vector pET11d (Pharmacia) in *E. coli* BL21 (DE3). The protein was initially purified on a Q Sepharose anion exchange column, eluting with a linear gradient of 0–1 M NaCl in 20 mM Tris-chloride pH 6.3. Further purification was achieved by a second ion-exchange step (Pharmacia MonoQ 10/10 column, linear gradient of 0–1 M NaCl in 20 mM Tris-chloride pH 8.3) followed by size-exclusion chromatography (Pharmacia Superdex 75 column, 100 mM NaCl, 20 mM Tris-chloride pH 8.3). The resulting protein was > 95% pure as judged by SDS–PAGE. The cDNAs coding for mutants of RhoGDI were prepared by site-directed mutagenesis using the polymerase chain reaction (PCR) with appropriate 'mutagenic' primers; these cDNAs were subcloned into pET11d and the protein expressed and purified as described above.

Recombinant Rac-1 was expressed in the expression vector pET11a (Pharmacia) in *E. coli* B834 (DE3). The protein was purified using an SP Sepharose cation exchange column; the protein was eluted with a linear gradient of 0–1 M NaCl in buffer B (20 mM sodium phosphate, 5mM magnesium sulphate, 10 mM DTT pH 7.4). Two peaks were eluted, at concentrations of 250 and 350 mM NaCl, corresponding respectively to truncated (1–188) and full-length Rac-1. A subsequent size-exclusion chromatography step (Pharmacia Superdex 75 column using 100 mM NaCl in buffer B) yielded > 95% pure protein.

NMR samples of RhoGDI were prepared in 50 mM sodium phosphate buffer pH 6.3 (90% H_2O, 10% $^{2}H_2O$), 1 mM DTT, 1 mM EDTA to final concentrations of between 0.5 mM and 0.8 mM. Samples of Rac-1 (GDP form) were prepared in the same buffer with the addition of 5 mM $MgCl_2$. *N*-acetyl *S*-farnesyl cysteine (Calbiochem) was dissolved in [$^{2}H_6$]-ethanol to a concentration of 35 mM; additions to the RhoGDI sample were made in 1 μl aliquots.

NMR spectra were acquired using a Bruker DMX500 or DRX600 spectrometer at a sample temperature of 25°C for the truncated RhoGDI(59–204) and 15°C for the full-length protein. A lower temperature was used for the second sample in order to reduce the rate of proteolysis of the full-length protein. The complete assignment of the backbone ^{1}H, ^{13}C, ^{15}N resonances of RhoGDI(59–204) at 0.5 mM was made using the HNCO, HN(CA)CO, HNCA, HN(CO)CA, CBCANH and CBCA(CO)NH experiments, whereas similar assignments of the backbone resonances of full-length RhoGDI at 0.8 mM

were carried out using the HNCO, HN(CA)CO, CBCANH and CBCA(CO)NH experiments. All these experiments were carried out in the gradient-enhanced sensitivity-enhanced mode. Processing of the three-dimensional triple-resonance data was carried out using Felix 95 software (Biosym Inc.). A semi-automated assignment strategy was employed using an in-house programme based on simulated annealing. [^{15}N–^{1}H]-TROSY spectra were recorded, using a gradient-enhanced and sensitivity-enhanced sequence [31], on a sample of 0.25 mM [^{2}H,^{15}N]-labelled RhoGDI with or without unlabelled Rac-1 at 15°C. To assess the solvent accessibility of residues of RhoGDI in the complex, microlitre aliquots of a stock solution of TEMPO (Sigma Chemical Co.) were titrated into the sample of the complex to give a final concentration of ~10 mM. ^{1}H chemical shifts are expressed relative to the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) at 0 ppm. The ^{13}C and ^{15}N chemical shifts were indirectly referenced to external DSS. Minimum chemical-shift changes of the amide resonances of RhoGDI-1 on binding Rac-1 were estimated as follows. The distances, in terms of ^{1}H and ^{15}N chemical-shifts ΔH and ΔN, from each cross-peak in the ^{1}H–^{15}N HSQC spectrum of the free protein to the nearest cross-peak in the complex were measured. The minimum chemical-shift difference was then expressed as:

$$\sqrt{\left(\frac{\Delta H}{0.03}\right)^2+\left(\frac{\Delta N}{0.3}\right)^2} \quad (1)$$

Values of this quantity of greater than 1.75 were considered significant.

The binding of Rac-1 to wild-type and mutant RhoGDI-1 was determined by measuring the decrease in the fluorescence of mantGDP (Molecular Probes Inc.) bound to Rac-1 on addition of RhoGDI, as described [32].

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